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Combined atomic force and fluorescence localisation super-resolution microscopy for imaging cells

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Abstract / Summary (100-150 words)

Fluorescence localisation microscopy techniques (STORM/PALM) and atomic force microscopy (AFM) are both capable of imaging subcellular features in physiological buffers at resolution well below the diffraction limit, making these techniques indispensable tools in cell biology research. As the type of information obtained by these techniques is very different -- AFM providing height and/or mechanical property maps of the sample surface and STORM the location of labelled biomolecules inside the cell -- the combination of these techniques in one setup has become desirable. This approach was previously hindered by the need to change buffers between imaging modes. By using a new fluorescent dye that enables STORM imaging in a buffer that is also compatible with AFM, we demonstrate correlative AFM and STORM on fixed cell samples without the need to change buffers. The use of photoswitchable fluorescent proteins, which offer another way to perform localisation microscopy without special buffers, is also demonstrated.

Article - 2500-3000 words (excluding biography, abstract etc.)

1. Introduction

Fluorescence microscopy and atomic force microscopy (AFM) are a powerful combination in providing different types of information that complement each other. Both are also compatible with physiological buffers, allowing the observation of biological specimen in their natural environment. Fluorescence microscopy allows the tagging of intracellular molecules and cellular components with high specificity, and their observation inside cells in a minimally invasive manner using non-destructive wavelengths of light in the visible spectrum. AFM, on the other hand, uses a sharp tip to measure the topography of the sample, or other physical properties of the surface such as adhesion or stiffness; see Figure 1. AFM can provide sub-nanometer axial resolution, or the tips can be functionalised to recognise specific molecules, but measurements are limited to the sample surface.

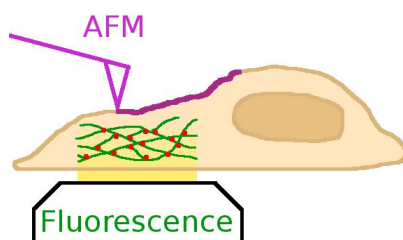


Fig 1: A schematic diagram showing the principles of atomic force microscopy (AFM) and fluorescence microscopy. AFM scans a sharp tip over the sample surface and produces a topographic image of the sample (magenta). With fluorescence microscopy, cellular components are labelled with fluorescent tags which light up (here green and red) when illuminated with certain wavelengths of light, allowing imaging inside cells.

Traditionally, the diffraction limit in light microscopy has restricted the resolution of fluorescence microscopy to about half of the wavelength of the fluorescence light. This is about two orders of magnitude more than the resolution of AFM, limiting the usefulness of correlative measurements. Recently developed superresolution microscopy techniques have brought the resolution of

fluorescence microscopy down by an order of magnitude to a few tens of nanometers, a similar scale to the typical lateral resolution of AFM when imaging soft biological samples.[1] Single-molecule localisation fluorescence microscopy techniques (such as STORM [2] and PALM [3]), in particular, have found widespread use in biological imaging, due to a relatively simple experimental implementation. In its most simple experimental form, direct STORM (dSTORM),[4, 5] the sample is illuminated with a high power laser while immersed in a reducing buffer which makes the fluorescent molecules blink.

The cyanine dye Alexa-647 has been widely reported as one of the best dyes for (d)STORM,[4, 5, 6] with relatively high brightness and good blinking statistics in a buffer containing an enzymatic oxygen scavenging system (GLOX) and a thiol such as cysteamine (MEA). Unfortunately, when AFM cantilevers are immersed in this buffer, some of the GLOX buffer components crystallise on the cantilever, making AFM image acquisition impossible. Previous works that combine AFM and STORM report changing the sample buffer between the imaging modes,[7, 8, 9, 10] but this can lead to movement and damage to the sample between the images.

In this work, we replace Alexa-647 by a similar cyanine dye, iFluor-647, which has excellent brightness, photostability and blinking properties in a buffer that contains MEA but no GLOX. We then combine AFM and fluorescence super-resolution microscopy in one setup for cellular imaging, without the need to change buffer between imaging modes.

The use of endogenous fluorescent proteins, such as the green fluorescent protein (GFP), offers an alternative to antibody labelling. With some fluorescent proteins -- called photoswitchable fluorescent proteins -- the on and off states can be controlled with specific wavelengths of light. Photoswitchable fluorescent proteins offer another way to perform localisation microscopy without special buffers. In this work we use a photoconvertible fluorescent protein mEOS3.2,[11] which usually emits in green with 488 nm excitation, but can also convert to yellow-emitting conformation upon 405 nm illumination. We combined mEOS3.2 with iFluor-647 labelling in HeLa cells, and demonstrate 2-colour localisation microscopy + AFM imaging in one setup.

2. Materials and Methods

2.1 Microscope setup

The combined AFM+STORM setup was built around a standard inverted microscope (Zeiss Axio Observer.Z1). The microscope was equipped with a LightHUB-6 laser combiner (Omicron, Germany) with LuxX 405 nm, 488 nm and 647 nm diode lasers (Omicron, Germany) and a JIVE 561 nm diode-pumped solid state laser (Cobolt, Sweden) for fluorescence excitation, an EMCCD (Andor iXon Ultra DU897) for fluorescence data collection, and a JPK Nanowizard 3 for AFM imaging. For localisation microscopy, the sample was illuminated and imaged from the bottom through a 100X NA 1.4 oil immersion objective (Zeiss Plan-Apochromat). For imaging iFluor-647, Zeiss filter set 50 was used (excitation 640/30 nm, dichroic mirror 660 nm, emission 690/50 nm), and for mEOS3.2 Zeiss filter set 31 (dichroic mirror 585 nm, emission 620/60 nm) with a ZET405/561x excitation filter (Chroma, VT) was used. The camera exposure time was set to 10-20 ms and EM gain to 600 with laser power at the sample $\sim 5 \text{ kW/cm}^2$. The camera pixel size at the sample plane was 145 nm, and the camera bit depth 16 bits. A total of 10,000 to 30,000 frames were acquired. The instrumentation for STORM data collection was controlled with μ Manager software.[12]

AFM imaging was performed with a SiN cantilever with a Si tip with nominal spring constant of 0.292 N/m, tip radius $< 10 \text{ nm}$ and gold coating on the reflex side (HYDRA-6V-200NG, Applied

NanoStructures, CA). Images were recorded on quantitative imaging (QI™) mode, which records a complete force-distance curve for each pixel without exerting lateral forces on the sample. For Fig 5 the set point was 4 nN and the scan time was 19 minutes for 512x284 pixel image with 800 nm ramp size and 8 ms pixel time, and for Fig 6 the set point was 3 nN and the scan time 15 minutes for 512x245 pixel image with 500 nm ramp size and 7 ms pixel time. The AFM images were processed by subtracting a 1st degree polynomial fit from each line.

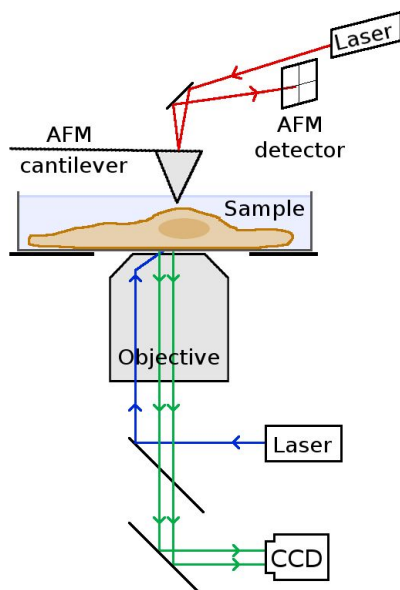


Fig 2: A simplified schematic diagram of the AFM+STORM microscope setup. The setup was built around a standard inverted microscope base, with an AFM to image the sample from top, and fluorescence setup underneath the sample for STORM imaging.

2.2 Sample preparation

HeLa cells were cultured at 37°C, 5% CO₂ in DMEM supplemented with 10% FBS, penicillin/streptomycin, and L-glutamine. The cells were plated on 35 mm dishes with #1.5 polymer coverslip bottom (ibidi, Germany) at seeding density of $\sim 1.5 \times 10^4$ cells per dish, and left to adhere for 16-24 hours. To unroof the cells, the medium was replaced with H₂O solution containing 10 µg/ml phalloidin and protease inhibitors (Roche) for 40 s, the cells were then flushed 10X and fixed for 20 minutes with 4% paraformaldehyde. For actin staining, dye-conjugated phalloidin stock solution (Alexa-647-phalloidin, Invitrogen, UK or iFluor-647-phalloidin, AAT Bioquest, CA) was diluted in 3% BSA in PBS, and the cells incubated for 1 hour in the dye solution. For tubulin staining, the samples were blocked for 30 min in 3% BSA in PBS, incubated for 1 h with anti-β-tubulin mouse antibody (T8328, Sigma) diluted 1:200 in 3% BSA in PBS, washed thoroughly, and incubated for 1 h with anti-mouse-iFluor-647 (16783, AAT Bioquest) diluted 1:500 in 3% BSA in PBS. For the HeLa cell line stably expressing mEOS3.2-lifeact, HeLa cells were transfected with a LNT/SffV-mEOS3.2-lifeact lentiviral expression construct following a protocol described in [13] using DMEM instead of RPMI-1640.

Stock solutions of MEA (1 M cysteamine (30070, Sigma-Aldrich) in H₂O, pH adjusted to 8.0 with HCl solution) and GLOX (0.5 mg/ml glucose oxidase (G6766, Sigma-Aldrich), 40 µg/ml catalase (C40, Sigma-Aldrich) in H₂O) were stored at 4°C and used within 1 week of preparation. The stock solutions were diluted in TN buffer (H₂O with 50 mM Tris pH 8.0 and 10 mM NaCl), supplemented with 10% w/v glucose if GLOX was added. GLOX stock was diluted 1:100, and MEA was used at final concentrations of 5-150 mM, typically 50 mM for imaging. The buffers were mixed immediately before use and added to the sample dish 15-30 minutes before imaging.

3. Results / Discussion

3.1. iFluor-647 characterisation

To test the performance of the iFluor-647 dye, HeLa cells were grown on dishes, fixed and the actin filaments were stained with either Alexa-647 or iFluor-647 conjugated phalloidin. Alexa-647 is a robust and popular dye for STORM imaging, with excellent brightness and switching properties in a STORM buffer containing oxygen scavenger. iFluor-647 is a similar dye which has recently been shown to be suitable for STORM imaging without oxygen scavenger.[14]

The molecular brightness of the dyes was measured as a function of the thiol (MEA) concentration in buffers containing either both enzymatic oxygen scavenger and the thiol (GLOX+MEA) or only the thiol (MEA only). It was found that the brightness of both dyes decreases with increasing MEA concentration in both buffers (Fig 3), but in very low MEA concentration (below ~20 mM) the dyes bleach quicker.[14] The optimal MEA concentration for imaging was found to be ~20-50 mM.

Importantly, the brightness of iFluor-647 in MEA only buffer was found to be comparable to the brightness of Alexa-647 in MEA + GLOX buffer. The localisation precision and thus the resolution of the final image increases with brightness, therefore the expected resolution of iFluor-647 in MEA only buffer is comparable to Alexa-647 in the MEA+GLOX buffer.

The image quality of both dyes was also tested in the different buffers. Figure 4 shows example STORM images of HeLa cells stained with Alexa-647 (top row) and iFluor-647 (bottom row) in buffers containing MEA+GLOX (left column) or MEA only (right column). While Alexa-647 yields a good image in MEA+GLOX buffer, in MEA only buffer the image quality of Alexa-647 is degraded, with bright spots from dyes molecules that are not blinking well (see Fig S3 in [14]) and the smallest details are not resolved due to lower localisation precision. iFluor-647, however, yields a good image in both buffers, and is thus suitable for correlative AFM + STORM imaging in MEA only buffer.

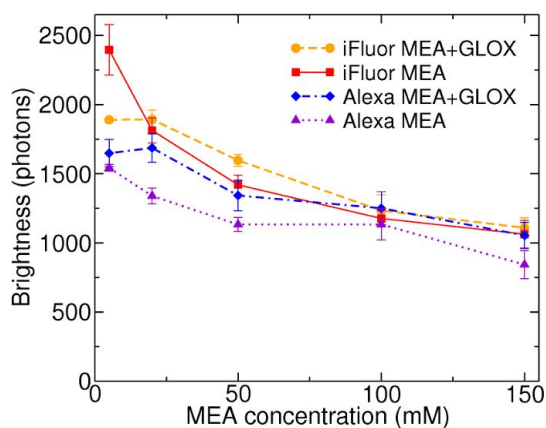


Fig 3: Mean molecule brightness of iFluor-647 and Alexa-647 phalloidin-conjugated dyes as a function of MEA concentration in buffers with and without enzymatic oxygen scavenger (GLOX). The localisation precision and thus the resolution of the final image increases with brightness. The brightness of iFluor-647 in MEA only buffer is comparable to the brightness of Alexa-647 in MEA + GLOX buffer.

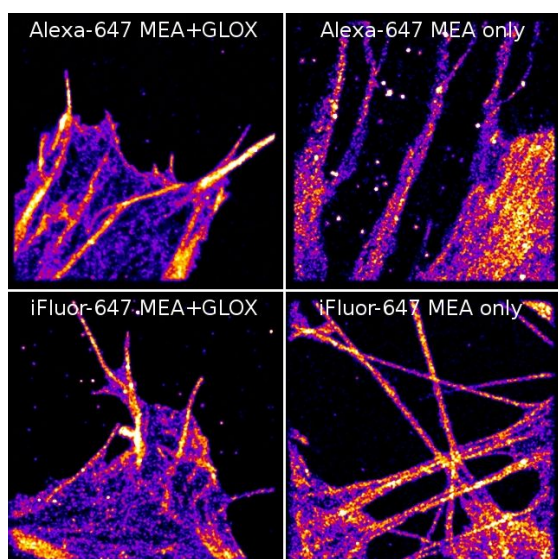


Fig 4: Example STORM images of HeLa cells stained with Alexa-647 (top row) and iFluor-647 (bottom row) in buffers containing 20 mM of the thiol MEA and enzymatic oxygen scavenger (left column) or 20 mM of the thiol MEA only (right column). In MEA only buffer the image quality of Alexa-647 is degraded, but iFluor-647 yields a good image.

3.2 Correlative AFM+STORM with iFluor

For correlative AFM+STORM imaging, HeLa cells were grown on dishes and the actin filaments were stained with iFluor-647-conjugated phalloidin. Before imaging the medium was changed to a buffer containing 50 mM MEA. STORM and AFM images were recorded one after the other, as simultaneous acquisition is not practical due to the overlapping spectrum of the imaging and AFM laser wavelengths, but since no buffer change is necessary, the images can be recorded in either order. Figure 5a-c shows an example of combined AFM + STORM imaging with iFluor-647, where the STORM image was recorded first and the AFM image directly afterwards, whereas in Figure 5d-f the AFM image was recorded first and the STORM image directly afterwards.

Some reports suggest that the AFM laser may bleach fluorescence in the red spectral region [7], but we found that the 850nm AFM laser in our system does not have significant effect on bleaching the 647nm excitable fluorophores, and the STORM image quality is not compromised if the AFM image is acquired first. On the other hand, some reports suggest that the STORM laser degrades the sample if the STORM image is acquired first so it could be beneficial to acquire the AFM image first [7, 9], however we found no evidence of sample damage after STORM imaging; it is likely that the sample damage observed in MEA+GLOX buffer is diminished in MEA only buffer. In any case, without buffer change the images can be acquired in whichever order is preferred.

The use of MEA only buffer also enables long term STORM imaging. Enzymatic oxygen scavenging in the normal STORM buffer changes the pH of the buffer over time, leading to detrimental changes in the dye molecule blinking properties as well as sample damage, and a typical maximum data acquisition time of ~2-3 hours before the buffer has to be changed. With MEA only buffer the pH change is eliminated, and therefore the imaging time is extended. The images Figure 5d-f were acquired after the sample had been kept in the microscope for >5 hours.

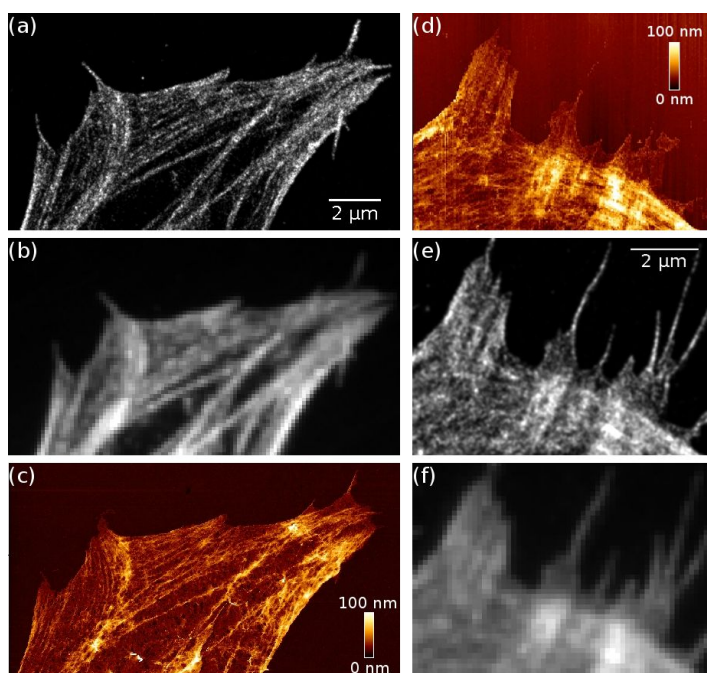


Fig 5: Correlative AFM+STORM images of unroofed HeLa cells stained with iFluor-647-phalloidin. (a) STORM, (b) wide-field fluorescence and (c) AFM images of a cell where the STORM image was acquired first, and the AFM image directly afterwards. (d) AFM, (e) STORM and (f) wide-field fluorescence images of another cell, where the AFM image was acquired first, and the STORM image directly afterwards.

3.3 AFM with two-colour localisation microscopy

For two-colour localisation microscopy + AFM, HeLa cells expressing mEOS3.2-lifeact were unroofed (i.e. the top membrane was removed, see Sample Preparation) and fixed, and tubulin labelled with iFluor-647. Before imaging the medium was changed to a buffer containing 50 mM MEA. Figure 6 shows an example of HeLa cells where the iFluor-647 (red) localisation image was acquired first, then the mEOS3.2 (green) localisation image, and the AFM scan was acquired directly after the localisation microscopy images without buffer change.

The use of endogenous fluorescent proteins, such as GFP, offers an alternative to antibody labelling. Both labelling methods can cause artefacts in biological imaging. Whereas antibodies can typically be used only in fixed cells, fluorescent proteins enable live cell imaging, and thus can help recognise artefacts caused by the fixation and permeabilisation process as well as nonspecific binding (and thus false localisation) caused by antibody labelling. With fluorescent proteins one of the most common problems is overexpression which can lead to false localisation and loss of function of the target protein. It would be useful to test both labelling strategies to check that the results obtained by both methods are in agreement. Combination of AFM with localisation microscopy can also be useful in recognising artefacts caused by fluorescence labelling and/or super-resolution image reconstruction.[10]

Since most photoswitchable fluorescent proteins emit in the green-to-yellow part of the spectrum, and the best STORM dyes usually in the red, the combination of these two labelling strategies for two-colour imaging is straightforward.

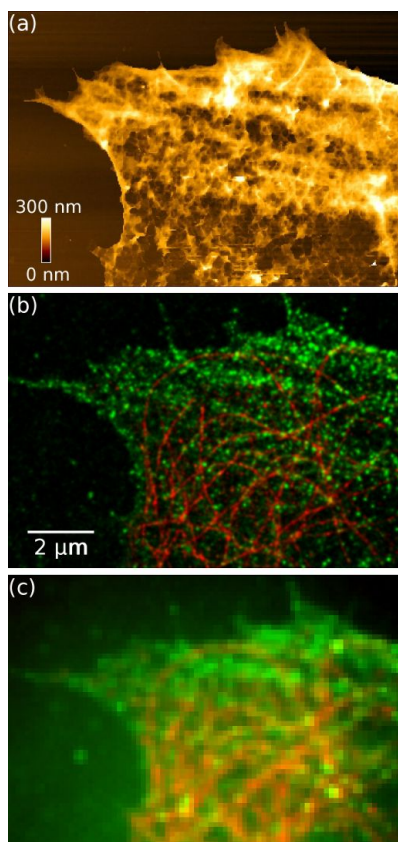


Fig 6: Correlative (a) AFM, (b) localisation microscopy and (c) wide-field fluorescence images of an unroofed HeLa cell. The cell was transfected with mEOS3.2-lifect (green colour), and tubulin has been immunolabeled with iFluor-647 (red colour).

4. Summary and Conclusions

Recent advances in both super-resolution microscopy and AFM have made combining these techniques a desirable tool for nanoscale biological research. A major drawback in combining AFM with localisation microscopy has been that the standard STORM buffer components, especially enzymes and glucose, stick to the AFM cantilever preventing AFM imaging. Previously, the combination of STORM and AFM has required a buffer change between the imaging modalities, [7, 8, 9, 10] which is cumbersome and leads to longer time intervals and possible movement and damage to the sample between the images. An alternative approach for localisation microscopy that avoids buffers with enzymatic oxygen scavengers is the use of quantum dots (QDs),[8] but their greater size of several nm in diameter can limit their use in labelling intracellular structures, and due to the long on-time of the QDs the sample has to be labelled sparsely.

We present an easy and straightforward method for correlative AFM + STORM imaging of fixed samples using iFluor-647 dye in a simple buffer containing the triplet quencher MEA but no oxygen scavenger. The use of MEA only buffer enables correlative imaging without the change of buffer between the imaging modalities, and allows the AFM and STORM images to be acquired in whichever order is desired. Another advantage of leaving out the oxygen scavenger is that the buffer does not degrade the sample over time, and we were able to acquire STORM and AFM images without compromised quality after keeping the sample in the microscope for >5 hours without any buffer change. We then combined iFluor-647 with the use of the photoswitchable fluorescent protein mEOS3.2 for correlative two-colour localisation microscopy + AFM.

The ability to perform high-quality STORM imaging without an enzymatic oxygen scavenger opens up exciting possibilities in correlative AFM and localisation microscopy. We aim to use this method for analysing the differences in images created with single-molecule localisation microscopy and AFM, and comparing different labelling strategies, such as the use of endogenous fluorescent

proteins, and data processing methods for artefact-free imaging. Besides correlative AFM+STORM imaging, STORM imaging in MEA only buffer can be useful for any application where longer term imaging of the sample is required, or sample damage caused by the STORM buffer is a concern.

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